Pseudomonas aeruginosa Short-Range Signaling Protein Influence On Biofilm Phenotypic Expression

A Thesis

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Abstract

Bacterial biofilms are communities of bacteria which grow on various surfaces. They are composed of the bacteria themselves and an extracellular polymeric slime (EPS) matrix which they encase themselves within. Biofilms can be found in any environment where there is persistent water, such as oceans, pipes, and the human body. Biofilms are formed when free-floating, planktonic bacteria attach to a surface and grow. Cells in a biofilm use communication via cell signaling bacteria to coordinate the behavior of the whole biofilm population for a diverse array of functions, including expression of virulence factors, proliferation and dispersal. In cell signaling, diffusible chemicals are released by individual bacteria which can be "sensed" by others in the biofilm.

A short-range cell signaling protein in *Myxococcus* was discovered to be responsible for social motility in biofilms. We have identified a single potential protein homolog in *Pseudomonas aeruginosa*, PA4079, for which we are testing to determine social motility differences between a wild-type strain of *P. aeruginosa* versus a strain with a knockout of the PA4079 gene. Biofilms of both a wild-type strain tagged with green fluorescent protein (GFP) and a PA4079 transposon knockout strain tagged with GFP are grown under various media and temperature conditions. Images have been collected to compare phenotypic differences among the biofilms. In current experiments, we are using time-lapse photography to determine if dispersal rates and motility are affected by the absence of this protein. We are also utilizing a flow cell system to manipulate biofilms under shear stress.

We believe the PA4079 gene and homologs may be responsible for dispersal from pathogenic biofilms and could represent a possible target for treating problematic biofilm infections. With

the information provided from this research, we will be able to better understand social motility and cell-signaling of *Pseudomonas aeruginosa.* Our results could bring forth new ways to treat and prevent pathogenic biofilms within orthopedic environments (i.e implants, reparative surgeries) as well as natural environments.

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I would like to express the utmost gratitude to Dr. Stoodley for allowing me the opportunity work in his group with little previous research experience. Learning the layout and protocols for working in the Center of Microbial Infection and Immunity, getting up to speed on the projects in the lab, and formulating my own experiment for an undergraduate research distinction thesis all in a year and a half was difficult. Dr. Stoodley guided me through the process, establishing a great advisor/student relationship, and assuring me that my work was headed in the right direction. I am extremely grateful for his time and effort, as he takes great pride in his work as well as his researchers' achievements; I hope prospective undergraduate researchers continue to have the opportunity and honor of working in his laboratory.

I would also to thank Dr. Matthew Swearingen for being an extraordinary mentor. He was willing to help me learn and develop my skills as a researcher, and provided me with guidance above expectation throughout my time in the laboratory. He was always willing to answer questions, give advice, and make sure I was putting forth my best work. He laid the groundwork for the majority of my undergraduate research experience, and I cannot thank him enough for everything he has done.

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1. Introduction

Bacterial biofilms are a leading concern in both the medical and environmental sciences. Biofilm formation, although known, is difficult to understand and diagnose which can lead to pathogenic bacterial infections. Planktonic cells adhere to any surface with consistent access to water, where they will grow and divide. These sessile bacterial communities encase themselves in a slime matrix composed of various extracellular polymeric substances (EPS) including polysaccharides, proteins, and DNA, providing them safety through structural durability and antibiotic resistance, where they will continue to grow and disperse throughout their environment.[1] Dispersal of biofilms in the body leads millions of people to experience chronic infections yearly (Figure 1).[2] *Pseudomonas aeruginosa* infections are most commonly found on catheters, wounds, contact lenses, implants, and also in cystic fibrosis patients as a non-surface-related infection.[1]

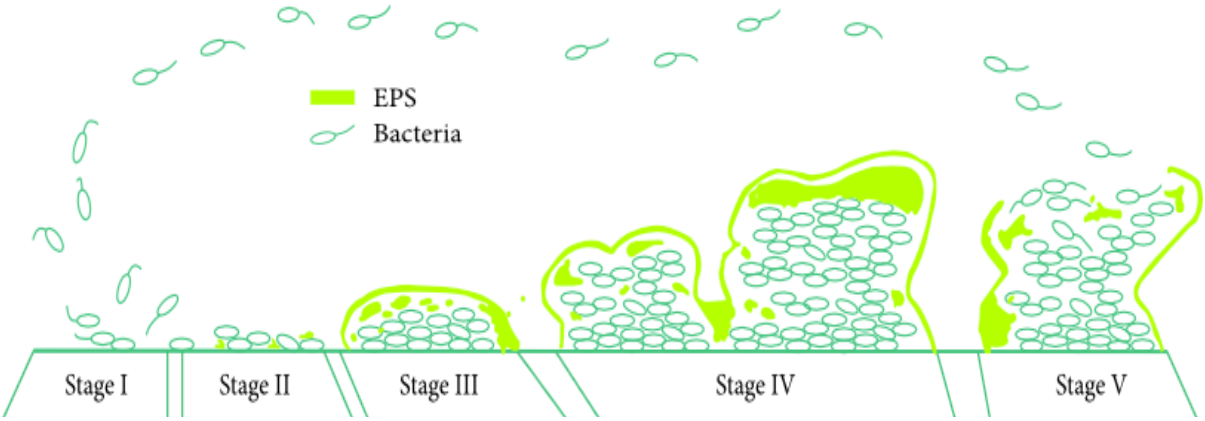


Figure 1[2]: Biofilm life cycle of *Pseudomonas aeruginosa.* Stage I: planktonic bacteria attach to an abiotic surface. Stage II: irreversible adherence of planktonic bacteria. Stage III: microcolony formation. Stage IV: biofilm maturation and growth of 3-D community. Stage V: dispersal of planktonic bacteria to other sites. Biofilm can adhere to biological and non-biological surfaces, and disperse throughout the body creating a chronic infection.

A short-range intercellular signaling protein was discovered in *Myxococcus xanthus* which was necessary for sporulation, gene expression, and cell motility. Termed C-factor, this protein was discovered by Seung K. Kim and Dale Kaiser in 1990.[3] It was seen that cell motility was a necessity for proper transmission of C-factor, and observations suggested that movement allowed cells to establish "spacial patterns" necessary for C-factor-dependent sporulation and gene expression. This spacial pattern caused *M. xanthus* cells to settle in the grooves of sandpaper-scored developmental surfaces with their long axes parallel to the grooves.[3]

In previous biofilm studies (Purevdorj-Gage *et al*, 2005), it was seen that *Pseudomonas aeruginosa* PAO1 biofilms form fruiting bodies within which cells differentiate into a planktonic phenotype, then disperse and swim into surrounding fluid. Upon dispersal, cells leave behind hollow mounds within the biofilm which do not exhibit further expansion or refill with new cell growth.[4] It was seen that a "critical cell cluster diameter of 100 μL" was necessary for swarming phenotypes to be seen.[4] This phenotypic differentiation suggested a regulated behavior/quorum sensing cell signal mechanism, for which a solid agar assay was developed (after Kim and Kaiser, 1990) by which the swarming phenotype could be initiated by growth on roughened agar, since swarming does not occur on conventional smooth agar. Results of this assay were seen to mirror the Kim and Kaiser results (1990), fueling further hypotheses regarding the C-factor.

From this analysis, a single potential C-factor homolog, which we have labeled PA4079, was identified in the PAO1 genome. This homolog had 50% amino acid identity to the C-factor of *M. xanthus*, and when compared to other C-factor homologs using Clustal W (http://www.ebi.ac.uk/clustalw/) this homolog was clustered with other biofilm-forming bacteria that exhibit social behavior, including *M. xanthus*. These clustered organisms also showed motility

through twitching, swimming, and swarming, which supported the hypothesis that the PA4079 gene and homologs may be responsible for dispersal from *P. aeruginosa* biofilms and could potentially represent a target for treating and preventing biofilm-related infections.

Assays were conducted previous to this investigation by Heather Eggleston to test for swimming, swarming, and twitching motilities between *P. aeruginosa* PAO1 (wild-type) and two mutants: one containing a transposon deletion and the other containing a clean deletion of the PA4079 gene. For twitching motility, bacteria were inoculated in the center of solid LB agar. For swimming motility, bacteria were inoculated in the center of LB, 0.2% agar plates. For swarming motility, bacteria were inoculated in the center of LB,1.5% agar plates. 1.5% agar is the standard for solidifying media for swarming motility assays.[5] All plates were incubated and grown for 4 days, and imaging was done daily to track the maximum motility zones for each test. Results in Figure 2 show that both mutant strains show higher levels of twitching (A), swimming (B), and swarming (C) motility when compared to the wild-type.

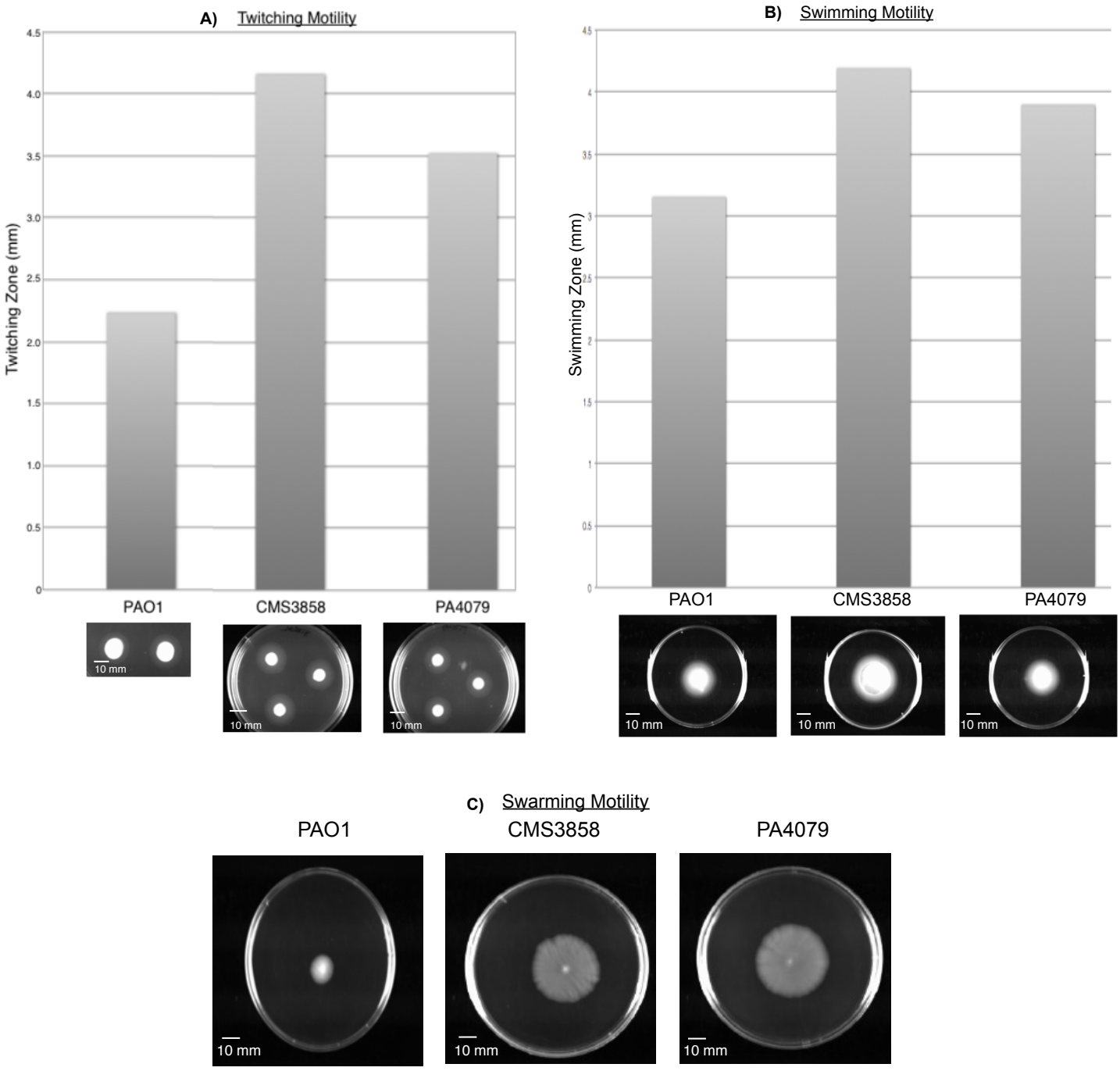


Figure 2: Motility Assay Results. **A, B)** Both PA4079 and CMS3858 mutants showed more twitching/ swimming motility than wild-type PAO1. **C)** More swarming motility was observed in both mutants, but no observable difference was noticed between mutant isolates.

The main aims of this investigation were to determine if social motility differences exist between a wild-type strain of *P. aeruginosa* when compared to strains lacking the PA4079 gene, and if the gene influences how biofilms phenotypically formed when influenced by various environmental conditions. The goal was confirm that PA4079 does affect motility rates within *P.*

aeruginosa biofilms. With the information provided from this research, we can better understand the social motility and cell-signaling of *Pseudomonas aeruginosa*. Our results could bring forth new ways to treat and prevent pathogenic biofilms within orthopedic environments (i.e implants, reparative surgeries) as well as natural environments.

2. Materials and Methods

2.1 Growth Curves for Strains

In order to investigate phenotypic differences involving growth rates and dispersal within biofilms, more information was needed to determine how each mutant isolate grew when compared to the wild-type. Isolates listed in Table 1 were provided by the University of Washington Genome Center (UWGC), including the wild-type strain PAO1, a mutant with a PA4079 gene knockout, a mutant with a clean deletion of the PA4079 gene, and each strain tagged with green fluorescent protein (GFP). Isolates listed in Table 2 were provided by Dr. Robert Shanks of the University of Pittsburgh, Department of Ophthalmology, including a mutant with a gene deletion for the SocA gene and manipulations of the CMS3858 mutant to reintroduce the PA4079 gene restore expression of the wild-type phenotype. Glycerol stocks were created for all acquired strains and stored at -80°C until needed for experiments.

For each isolate, 5 mL Lysogeny Broth (LB) broth or LB supplemented with gentamicin (30 µg/mL) was inoculated to grow liquid cultures overnight at 37°C. 5 µL culture was diluted in 5 mL fresh media, then the dilutions were dispensed in 200 µL increments into a BD Falcon 96-well polystyrene plate (Image 1) in four replicates. 24-hour kinetic reads were taken using a spectrophotometer set to OD_{600} at 37°C. For all initial dilutions per strain, serial dilutions were conducted at the initial (0-hour) and 24-hour time points to calculate CFU/mL for each strain to determine if strains grow at similar rates in under the same conditions (Table 1A).

Strain #	Description	Notes
PAO1	PAO1 (WT)	Wild-Type
PA4079	PAO1 ΔPA079	PAO1 with PA4079 gene knockout
CMS3858	PAO1 ΔPA079	PAO1 with a clean deletion of PA4079
PAO1 + pMRP9*	PAO1 + pMRP9	PAO1 tagged with GFP
PA4079 + pMRP9*	PAO1 ΔPA079 + pMRP9	PA4079 tagged with GFP
CMS3858 + pMRP9*	PAO1 ΔPA079 + pMRP9	CMS3858 tagged with GFP

Table 1: Isolates acquired from the University of Washington Genome Center.
* Isolates were tagged with GFP via plasmid (from Dr. Mike Franklin, Montana State University) in our laboratory.

Strain #	Description	Notes
CMS4202	PAO1 ΔPA2931	PAO1 with SocA gene deletion
CMS4292 **	PAO1 ΔPA079 + pMQ132	CMS3838 + empty plasmid (control)
CMS4293 **	PAO1 ΔPA079 + pMQ539	Gene compliment to restore WT function CMS3858 + pMQ132 + PA4079
CMS3960 **	PAO1 ΔPA079 + pMQ400 (tdtomato)	CMS3858 tagged with RFP isolated from tomato

Table 2: Isolates acquired from Dr. Robert Shanks, used only in crystal violet assay.
** Isolates require 30 µg/mL gentamicin in media for growth.

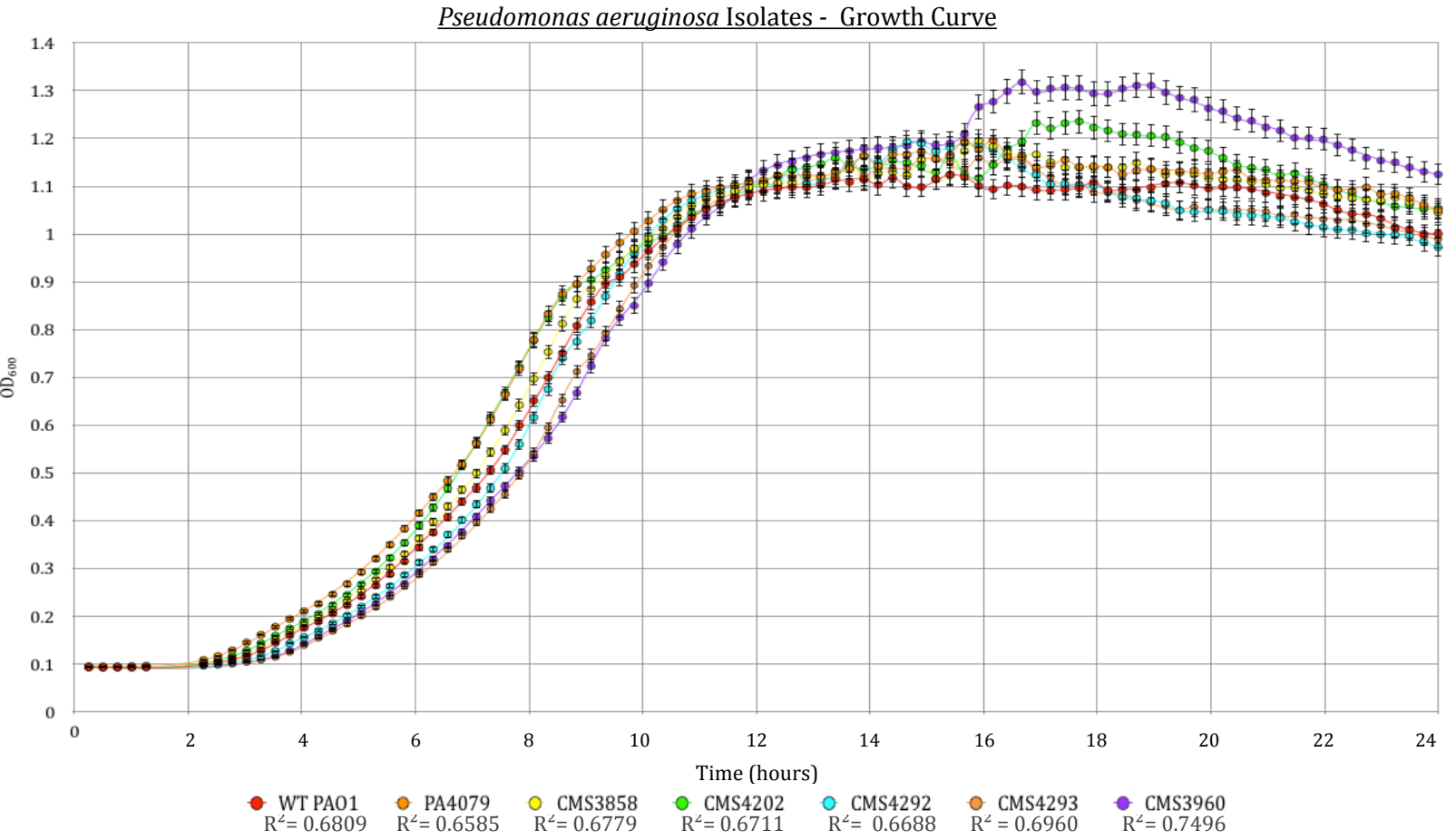


Figure 3: Growth curves for both all strains at 37°C show no significant differences in growth rates between initially acquired strains with temperature changes. Mutations do not appear cause a difference in growth rate. Error bars calculated using standard deviation calculations.

2.2 Crystal Violet Assay

A crystal violet assay was conducted to get quantifiable data for the amount of biomass produced by each strain over time. Isolates from Tables 1 and 2 were grown in LB broth or LB broth supplemented with 30 µg/mL gentamicin, depending on the required growth conditions of each isolate, overnight at 37°C. Cultures were plated in 2 Costar 96-well non-coated polystyrene plates, one for Day 3 tests and one for Day 5 tests, and in 35 mm polystyrene petri dishes for use with confocal microscopy. Established biofilms were rinsed with 10 mL sterile phosphate-buffered saline (PBS) solution (Thermo Fisher) daily before adding fresh media. On days 3 and 5 for respective biofilm growth, 180 µL of spent media and planktonic cells were removed from

each well of the 96-well plate. Biofilms were rinsed once with 180 µL sterile PBS solution, inverted, and tapped dry on filter paper. 200 µL of 0.1% crystal violet staining solution (from 1% stock in distilled water) were added per well, then incubated at room temperature for 15 minutes before being inverted and tapped dry on filter paper. Wells were rinsed with 200 µL sterile PBS solution to remove excess crystal violet solution and inverted to remove sterile PBS. 200 µL of 95% EtOH solution was added per well and allowed to incubate on the bench at room temperature for 10 minutes. After incubation period, absorbance readings were taken at OD_{570} and OB_{595} to compare biofilm formation (Figure 4B)

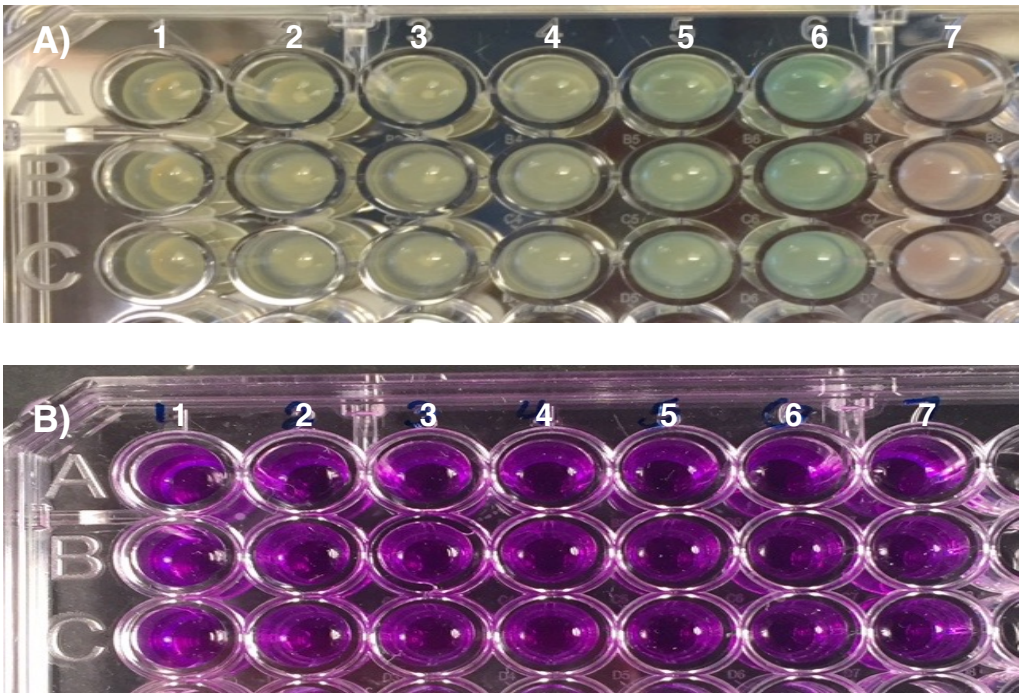


Figure 4: Crystal violet assay plate, **A)** pre-readings, **B)** post-readings. Cultures plated in triplicate. 1: WT PAO1, 2: PA4079, 3: CMS3858, 4: CMS4202, 5: CMS4292, 6: CMS4203, 7: CMS3960.

2.3 Biofilm Production - Static Growth

For all biofilm experiments, only isolates tagged with GFP were used to visualize differences between the strains; biofilms were counterstained with propidium iodide to compare live/dead cells before imaging. Propidium iodide (PI) was chosen as a counterstain for these biofilms because it will only permeate the cell membrane of dead cells, therefore live cells appeared bright green and dead red under GFP and RFP filters respectively. To understand how to build, establish, and properly image biofilms, static biofilms were grown in 35 mm MatTek glass-bottomed microwell dishes (14 mm microwell). Glass-bottomed dishes were chosen for experimental purposes, although *P. aeruginosa* has been seen to establish better adherence to hydrophobic materials like coated polystyrene, so biofilm progression could be visualized and captured.[6,7]

2.3.1 Temperature Variation

Biofilms were grown at two temperatures, 37°C and 22°C, to determine if temperature difference influenced rate of establishment and phenotypic expression of cells within the biofilms. Trials were conducted for 5 days at each temperature. MatTek dishes were set up for PAO1, PA4079, and CMS3858 in two replicates, with 30 µL liquid culture in 3 mL LB broth per dish. Established biofilms were rinsed with 10 mL sterile PBS solution daily before the addition of 2 µL PI and imaged. 3 mL fresh media was added per dish before being returned to proper incubator overnight.

2.3.2 Media Variation

Biofilms were also grown in M9 minimal media supplemented with 2% glucose to determine if nutrition played a role in biofilm establishment and phenotypic expression of *P. aeruginosa*. MatTek dishes were set up for PAO1, PA4079, and CMS3858 in duplicate with same procedures used for temperature variation experiments. Biofilms were grown at both 37°C and 22°C for a total of 7 days.

2.4 Biofilm Production - Flow Cell System

Biofilms were grown using a flow cell system to determine if these mutants establish biofilms under dynamic conditions, and how the cells behave within biofilms when experiencing hydrodynamic flow conditions. Flow cell systems allow biofilms to grow and mature as planktonic cells are removed by the flow of fresh media.[8] Using the flow cell system not only allows for the *in vitro* cultivation and evaluation of *P. aeruginosa* biofilms, but also provides a way to non-invasively observe the growth,
physiology, and structure of the bacteria when paired with microscopy.[8,9]

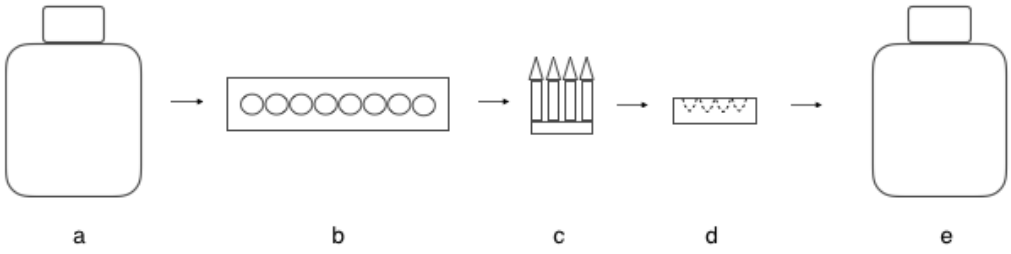
2.4.1 Construction and Assembly

Flow cell systems used for the following experiments were constructed using a metal flow cell (Biosurface Technologies Corporation) with a mounted microscope slide holding 4 borosilicate microglass cells, each 1.00MM I.D. x 0.15MM Wall x 50 Length, (Friedrich & Dimmock, Inc.) as seen in Figure 5.

Figure 5: Image of flow cell cassette used in each biofilm experiment. Numbers 1-4 on left side refer to lines coming from bubble trap and multi-channel pump. Strain numbers on right side indicate which strain type is growing in which lines, also showing that all trials run with this flow cell were done in duplicate for each strain. Each glass cell measures 10 cm long. Size 13 silicone tubing attaches directly to the inlet and outlet of each cell as shown, where it is attached to an additional 50 cm of size 13 tubing after and before inoculation and collection ports respectively.

Systems were comprised of an autoclavable inlet media vessel (Nalgene Company) holding appropriate sterile media for each trial of biofilms grown, glass windows and condensers to monitor flow rate and check for contamination, plastic and stainless steel line clamps to prevent contamination during inoculation, a high-precision multichannel pump (Ismatec) to drive the flow of the medium through the system, bubble traps, rubber inoculation and collection ports, the flow cell cassette (Figure 5) within which the biofilms are cultivated and subsequently viewed, and an outlet waste container (Nalgene Company). These individual components were connected by peroxide-cured silicone tubing (Masterflex) and various plastic and T- connectors (Cole Parmer). All tubing from the inlet media vessel before the pump and after the collection ports to the outlet waste container was size 16 Masterflex tubing, ID 3.1 mm. At the multichannel pump smaller Masterflex tubing, ID 0.8 mm, was substituted to connect the bubble trap, rubber ports, and flow cell (Figure 6).

A)



B)

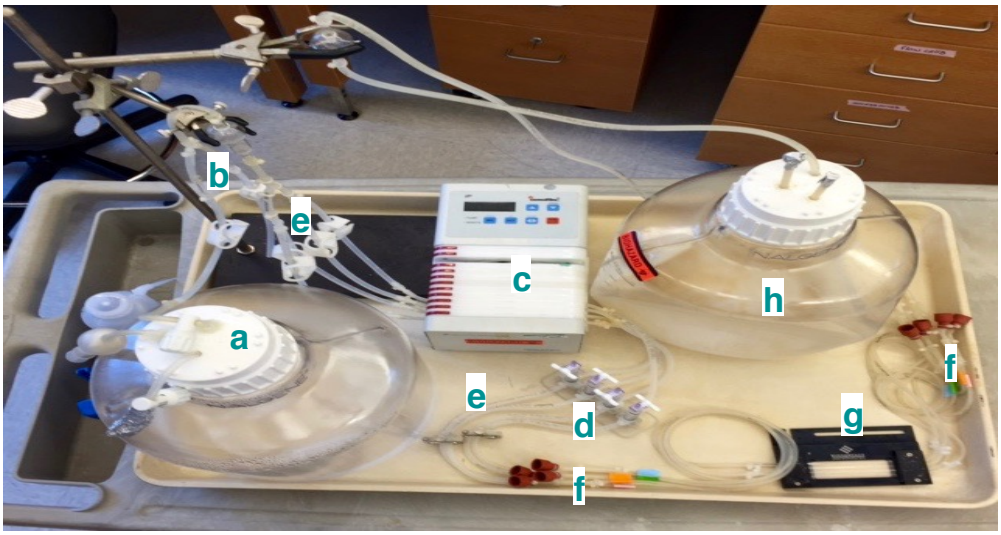


Figure 6: A) Schematic diagram of a flow cell system including: a. medium container, b. multichannel pump. c. syringe bubble trap; d. flow cell; e. waste container connected using silicone tubing providing a closed system. Arrows represent the direction of flow. **B)** Aerial view of actual flow cell setup used for experiments. a: media tank, b: glass windows and condenser, c: multichannel pump, d: syringe bubble trap, e: plastic/stainless steel line clamps, f: rubber ports, g: flow cell, h: waste container.

Precautions were taken to minimize air bubbles from forming within the system and disturbing the biofilms. These precautions included sealing around tubing at the inlet medium vessel with silicone caulking to make sure it was airtight, fitting the vessel with a venting apparatus filled with glass wool for exhaust, the use of an elevated back flow-preventing condenser before the pump to draw air bubbles out of solution by reducing the negative pressure gradient within the tubing, and the use of a bubble trap made up of sterile 5 mL Luer-Lok tip syringes (Becton Dickinson) and plastic stopcocks to capture any remaining air bubbles downstream from the multichannel pump before they could enter the flow cell cassette (Figure 7).

Figure 7: Bubble trap setup for dynamic growth biofilm experiments. Incorporation of the bubble trap downstream from the multi-channel pump prevented air bubbles introduced into the media from destroying biofilm growth in the flow cell.

Once assembled, liquid media for the current experiment was mixed in the inlet media vessel before the entire system was autoclaved for sterilization (121°C, 55 minutes) with all line clamps open. Upon removal from the autoclave, the inlet media container was connected to the rest of the silicone tubing under sterile conditions, all line clamps were closed, and stopcocks were added to the syringe tips. The system was allowed to cool to room temperature overnight to check for contamination, then clamps were opened as the pump was run at maximum speed to saturate the tubing for inoculation. Saturating the tubing allowed us to check for leaks and/or blockage within the system the following day. At this time, the pump was calibrated to flow at a rate of 100 μL/min, 0.51 rpm. If an experiment was to be conducted at 37°C, the entire system

would be placed in a large incubator after tubing was saturated and checked while in the incubator for leaks; if experiment was to be conducted at room temperature, approximately 22°C, the system would remain on a cart within the laboratory area for the duration of the trial.

2.4.2 Process for System Inoculation/ Extraction of Effluent

For all flow cell experiments, the two strains of *P. aeruginosa* used were PAO1 (wild-type) and the CMS3858 mutant (clean deletion of PA4079 gene). Liquid cultures for each strain were grown overnight in the proper media for the trial at 37°C. A 10^{-2} dilution was prepared with fresh media for all overnight liquid cultures prior to inoculation. Before inoculating flow cell system, tubing was clamped off roughly 15 cm upstream the flow cell cassette using the stainless steel clamps seen in Figure 6 (label e). Inoculation ports were sterilized with 70% EtOH solution before each inoculation. Using sterile 5 mL Luer-Lok tip syringes (Becton Dickinson) and 22 gauge 1½ inch needles (Becton Dickinson) seen in Figure 8A, lines 1 & 2 were inoculated with 5 mL of the wild-type PAO1 dilution and lines 3 & 4 were inoculated with 5 mL of CMS3858 dilution. The system was seeded at a rate of 20 drops per minute to prevent cell lysis or disruption of previously-seeded lines. Figure 8B shows how inoculations were carefully conducted to not create air bubbles within the system. Lines were left clamped shut overnight to allow bacterial cells to adhere within the glass cells, then lines were unclamped and media flow was initiated the following day.

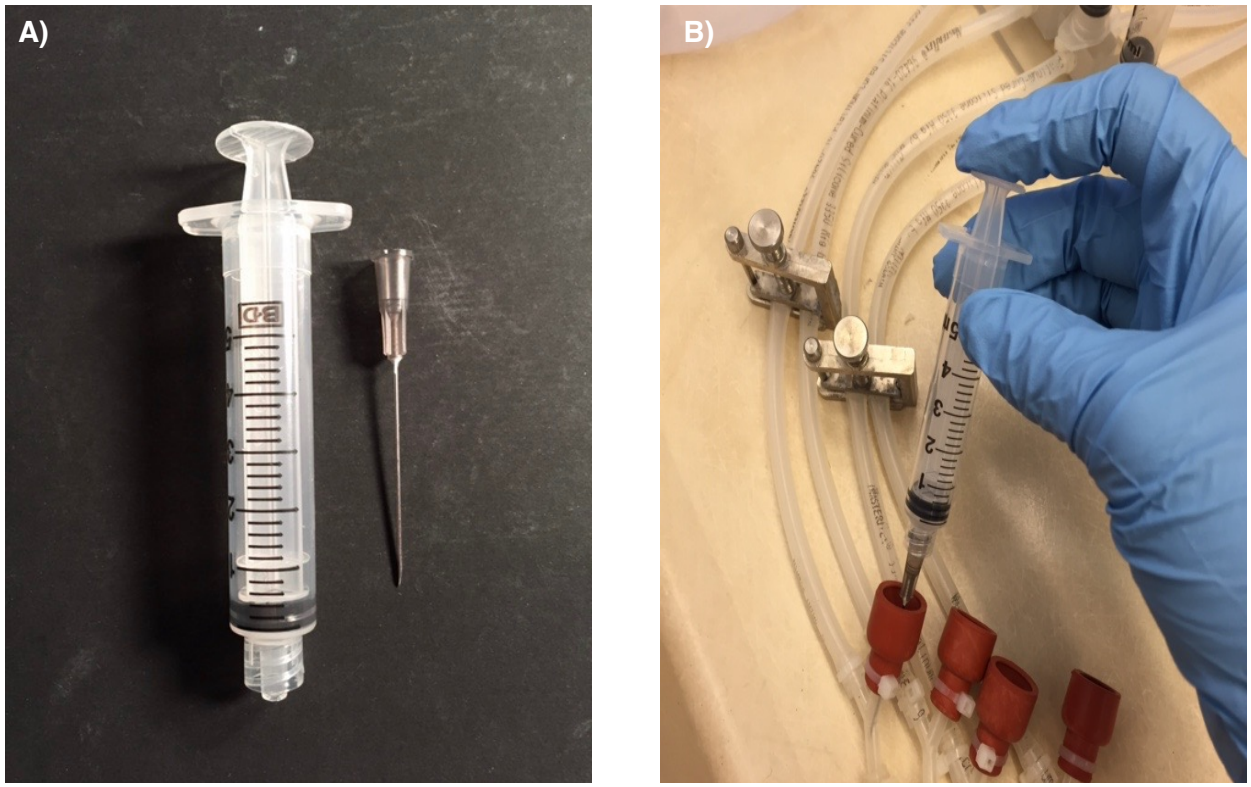


Figure 8: Inoculation of the flow cell. Before inoculating, tubing was clamped 10 cm upstream of the flow cell, plastic line clamps upstream the multi-channel pump were clamped as another level protection from contamination. **A)** The flow cell system was inoculated using a sterile 5 ml syringe and accompanying 22 gauge 1 ½ inch needle (Becton Dickinson). **B)** The inoculation port was filled with 70% EtOH solution, pierced through the center until the needle was visibly within the y-shaped junction, then 5 mL inoculum was seeded into the line at 20 drops per minute to prevent disruption of previously-seeded lines.

Once inoculated, 200 µL of each inoculum was placed in the first wells of a 96-well polystyrene plate (Falcon) for serial dilution and enumeration to calculate how many CFU/mL were in the inocula. Inoculum was diluted (20 µL into 180 µL LB broth) to a dilution factor of 10^{-8} down the corresponding column in the plate, then 10 µL (4 sets of 2.5 µL) were spotted on LB agar before placed in a 37°C incubator for enumeration the following day. Daily extractions of effluent followed the same process for serial dilution and enumeration, where 200 µL effluent was slowly drawn from each line from the rubber collection ports sterilized with 70% EtOH using 1 mL tuberculin slip-tip syringes (Becton Dickinson), seen in Figure 9A. Effluent was placed into the first wells of the 96-well plate and serial dilution and enumeration was conducted

as with inoculation. Extractions, serial dilutions and enumerations were completed daily for each line during each experiment to ensure biofilms were growing at proper rates and confirm no contamination had occurred within the lines.

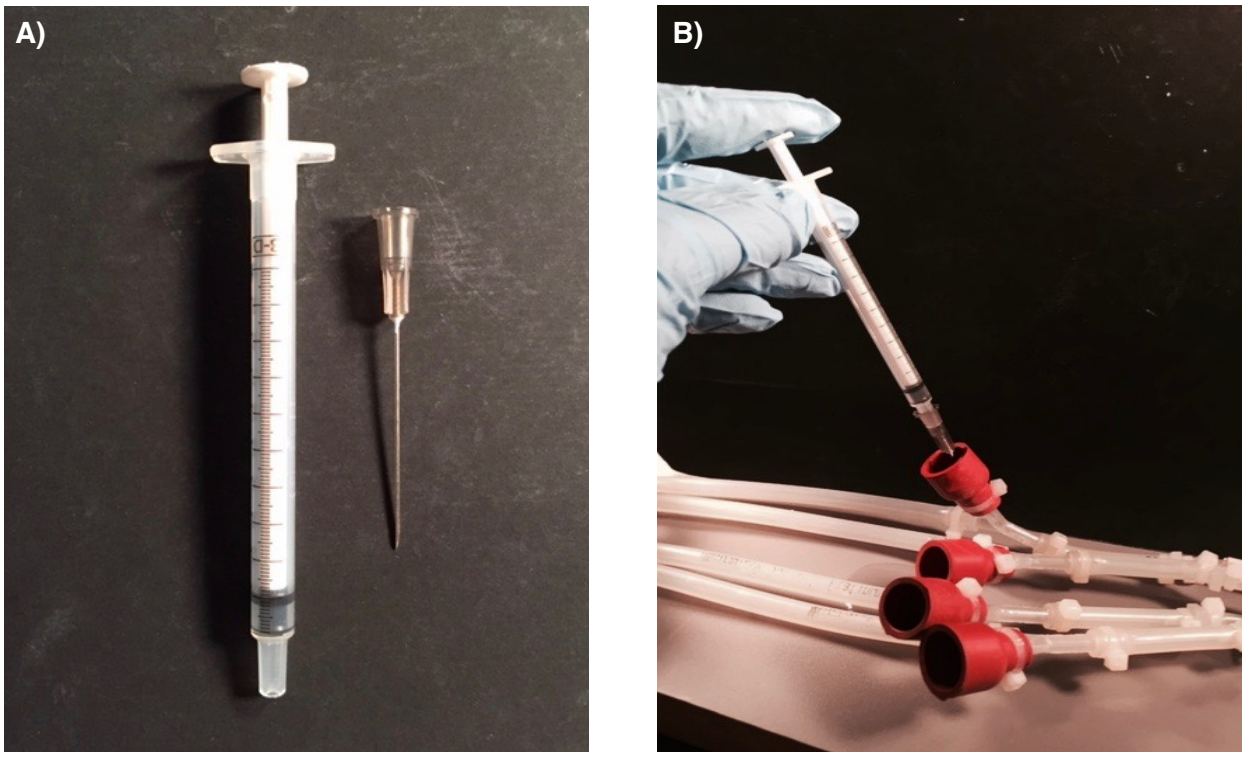


Figure 9: Procedure for Drawing Effluent. Effluent draws were conducted daily before imaging to calculate CFU counts and ensure biofilms were growing as expected. **A)** Effluent was drawn using a sterile 1 ml syringe and accompanying 22 gauge 1 ½ inch needle (Becton Dickinson). **B)** As with inoculation, the collection port was filled with 70% EtOH solution, pierced through the center until the needle was visibly within the y-shaped junction, then 200 µL inoculum was drawn off slowly per line to prevent disrupting other biofilms. Process was repeated for each line.

As with the static growth experiments conducted in MatTek dishes, biofilms were grown at two temperatures, 37°C and 22°C, to determine if temperature difference influenced rate of establishment and phenotypic expression of cells within the biofilms during dynamic growth. Trials were conducted for 5 days for both 37°C and 22°C. For the 37°C growth experiment, the system only left the incubator when lines were being imaged for observation.

2.4.4 Media Variation

Similar to the static growth experiments conducted in MatTek dishes, biofilms were grown using either full-strength LB broth (25g LB broth miller per 1L dH_2O) or 1/10x strength LB broth (2.5g LB broth miller per 1 L dH_2O) to determine if starvation and nutrient levels play a role in biofilm establishment and phenotypic expression of *P. aeruginosa*. MatTek dishes were set up for PAO1, PA4079, and CMS3858 in duplicate with same procedures used for temperature variation experiments. Biofilms were grown in both media types at 22°C for 5 days, and for full strength LB broth at 37°C for a total of 5 days.

2.4.5 Disassembly and Cleaning

At the conclusion of each experimental trial, established biofilms were flushed out of the glass cells and tubing before the next system was ready to be set up and autoclaved. The inlet media vessel was clamped off, and another piece of connected tubing was opened and set up for bactericidal solutions. 50% bleach solution was run through the system at maximum pump speed for approximately 30 minutes, then 70% EtOH solution was substituted in and run through for 15 minutes. Tubing was detached, discarded, and replaced after each trial as needed. Downstream effluent tubing was flushed with UltraPure DNAse/RNAse-free distilled water (Thermo Fisher) at maximum pump speed and massaged as needed to dislodge any remaining adherent biofilm waste matter. The flow cell was carefully opened and the glass microscope slide was removed from the flow cell base along with any cracked or broken glass cells. Once the entire system was checked over to ensure no leaks were imminent, the waste container then received 1 L of 50% bleach solution and was allowed to destroy any living bacteria overnight.

The following day, all liquid was pumped out of the system at maximum pump speed, waste vessel contents were autoclaved and disposed of in the proper biohazard waste container, and the media and waste vessels were thoroughly washed before reassembly; the system was then autoclaved and recycled for the next experiment.

2.5 Pyocyanin Assays

P. aeruginosa is known to produce the distinctive blue pigment pyocyanin, which appeared to be hyper-expressed within the mutant isolates when grown on solid LB agar or in LB broth. Our group was interested to see if pyocyanin production levels had correlational value to the presence or absence of the C-factor. Previously, *P. aeruginosa* has been seen to not produce pyocyanin if grown in culture media containing >1% glucose in solution, even in medias supplemented with blood or veal infusions.[10]

All strains used in pyocyanin pigment assays were not tagged with GFP to prevent mistaking pyocyanin production and fluorescence for GFP. Various tests were set up and conducted to determine whether or not the increase in pigment could be contributed to the loss of the PA4079 gene. Initial assays using LB broth and agar plates were conducted to get a basic understanding of how much pyocyanin was being produced by the wild-type PAO1 and two mutant strains. Liquid cultures for PAO1, PA4079, and CMS3858 were grown in 5 mL LB broth overnight at 37°C. Photographs were taken of all liquid cultures for visual comparison of pyocyanin production (Figure 10A).

In assay 1, 1 mL of each liquid culture was deposited into labeled 1.5 mL microcentrifuge tubes before being spun down at maximum speed for 1 minute. 1.5 mL of each liquid culture

was deposited into cuvettes before imaging for visual comparison of the supernatant pigment (Figure 10B). 100 µL of supernatant was then placed into the wells of a 96-well microtitre plate, then placed in a GelDoc UV gel documentation system to get an image of the pyocyanin fluorescence (Figure 10C).

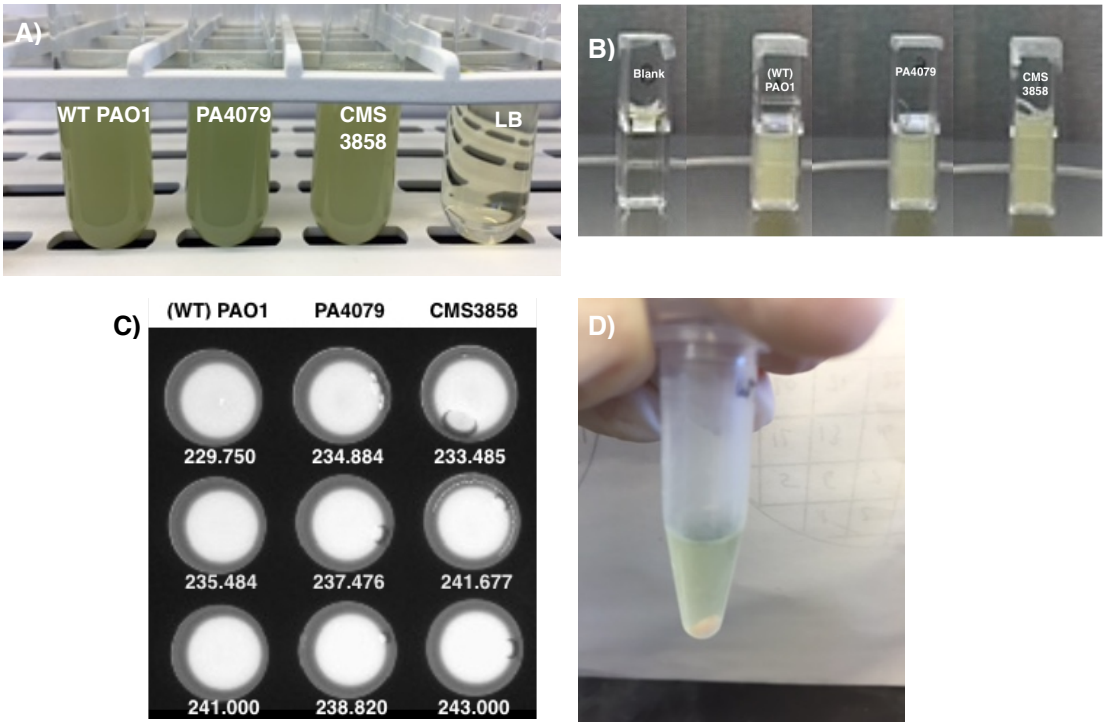


Figure 10: Pyocyanin Production. 1: WT PAO1, 2: PA4069, 3: CMS3858, B: Blank LB broth for control. **A)** Liquid cultures immediately after removal from incubator. **B)** Cuvettes containing 1.5 mL liquid cultures. **C)** GelDoc image of supernatant pyocyanin fluorescence analyzed with FIJI. **D)** WT PAO1 post-centrifugation. Can distinguish pyocyanin in supernatant (green) from cell pellicle (pink/tan).

For assay 2, Pseudomonas isolation broth and Jensen's media were made to determine if nutrition could influence pyocyanin production. Liquid cultures for PAO1, PA4079, and CMS3858 were grown in 5 mL of each media (6 liquid cultures total) overnight at 37°C. The following day, 1 mL per liquid culture was deposited into individually-labeled 1.5 mL microcentrifuge tubes before being spun down at maximum speed for 1 minute. Like in assay 1, 100 µL of supernatant was drawn off from each spun-down culture, then placed in a black-

walled 96-well plate to take absorbance readings. Images were once again taken using the GelDoc system as in assay 1.

2.6 Microscopy and Data Analysis

Photographs were taken using a smartphone camera (iPhone). Images of statically grown biofilms were captured using an EVOS® imaging microscope with both transmitted light imaging and GFP/RFP filters. Images for dynamically grown biofilms were completed using a Leica microscope paired with Micro-Manager Open Source Microscopy Software and time-lapse photography to detect phenotypic differences between the strains; the Leica microscope was also used other general microscopy needs throughout research tenure.

Scatterplots and R^2 values were generated using Excel. In graphs and charts, standard deviations were calculated and represented by error bars. FIJI (Fiji Is Just ImageJ) software was used to track the speed of individual cells from time-lapse photography image stacks. All tracking was done manually, for which the potential of error has been accounted for by the software.

3. Results

3.1 Crystal Violet Assay

The crystal violet assays showed little difference between the mutants and wild-type in the amount of biomass produced by the strains. Figure 11 shows the relationships seen from assays conducted on Days 3 and 5 of growth respectively.

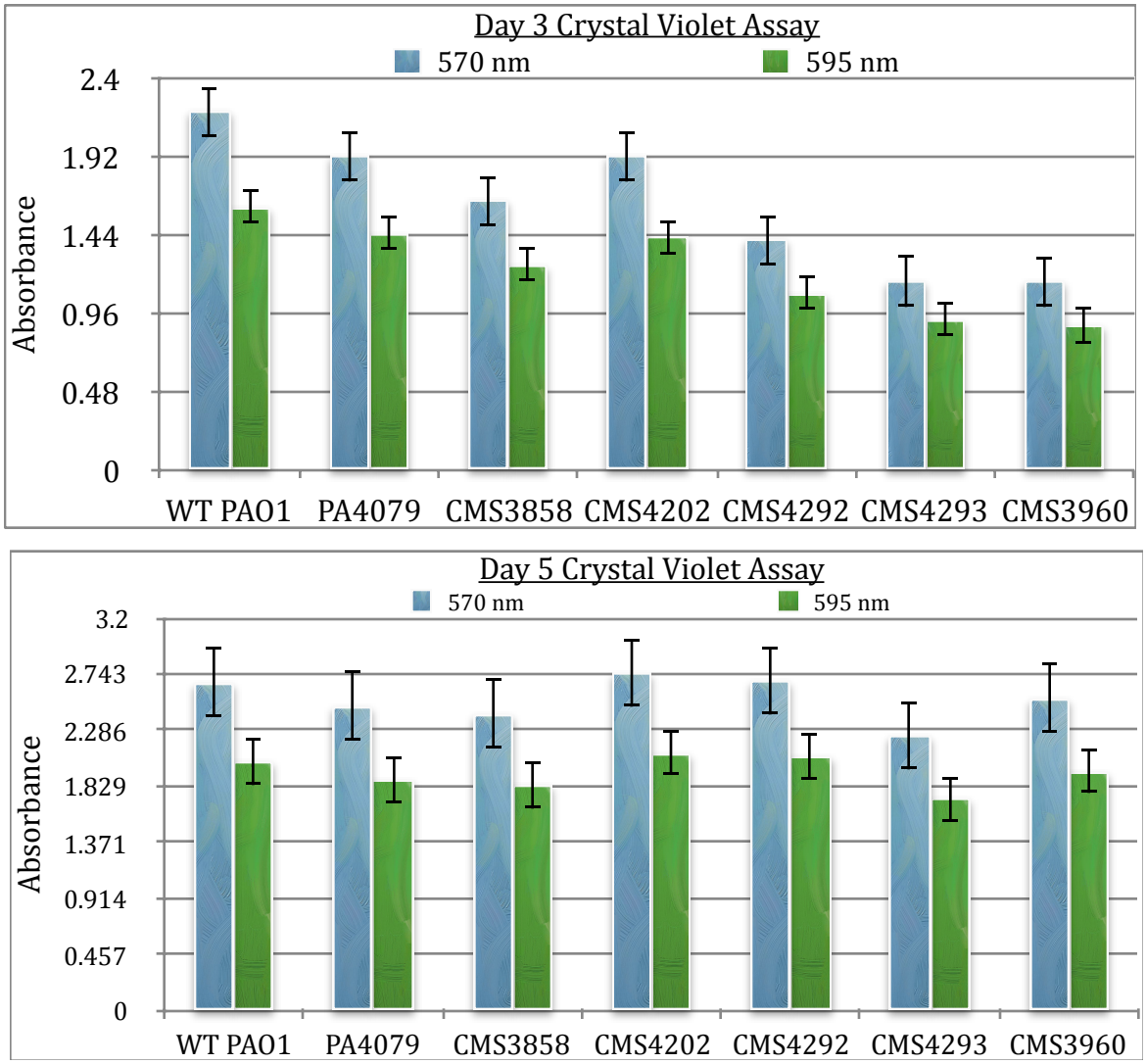


Figure 11: Crystal violet assay results. On both Day 3 and 5 at 570 and 595 nm, no major differences could be observed in biomass production between WT PAO1 and mutants.

3.2 Static Biofilm Growth - Dishes

3.2.1 Growth at 37°C - Varied Media

When grown in LB broth, no noticeable differences were initially observed between GFP-tagged and non-GFP strains in either wild-type or mutants when grown at 37°C. On Day 2, PA4079 showed signs of more tower formation and live attachment to the edges of the glass coverslip, and CMS3858 showed more signs of more motility which led to difficulty in imaging. By Day 4, the mutant biofilms expressed a filamentous, thread-like phenotype with this experiment trial (Figures 12A and 12B). Wild-type PAO1 showed a consistent biofilm lawn throughout the entire experiment (Figure 12C).

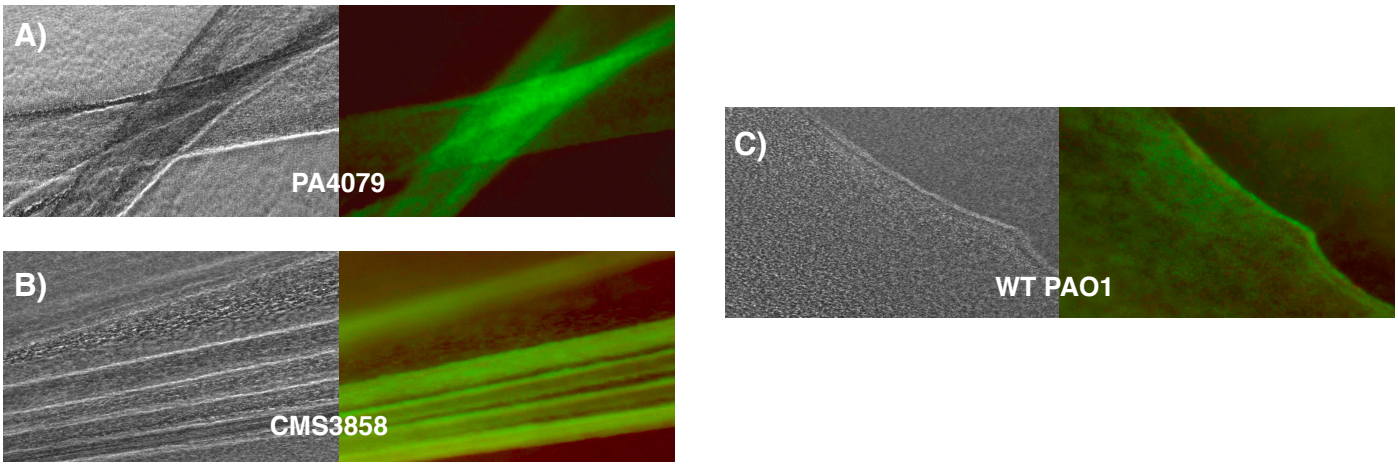


Figure 12: Static Biofilm Growth Results - LB Broth. All photos were taken under regular transmitted light (left) and GFP/RFP fluorescent light (right) **A)** Day 4 PA4079 biofilm formation. **B)** Day 4 CMS3858 biofilm formation. **C)** Day 4 wild-type PAO1 biofilm formation.

When grown in M9 minimal media supplemented with 2% glucose, small string-like structures were observed in the wild-type PAO1 biofilm throughout experiment; filaments were finally stained red on Day 5 of the trial (Figure 13A). Cells in each of the mutant biofilms were more “clumped” to form an aggregate-like biofilm, where cells in the wild-type PAO1 biofilm

were dispersed normally throughout the entire experiment (Figure 13B). Days 6 and 7 did not show any major differences in biofilm expression, so future experiments using minimal or lower-strength medias were ended on Day 5 as with LB broth experiments.

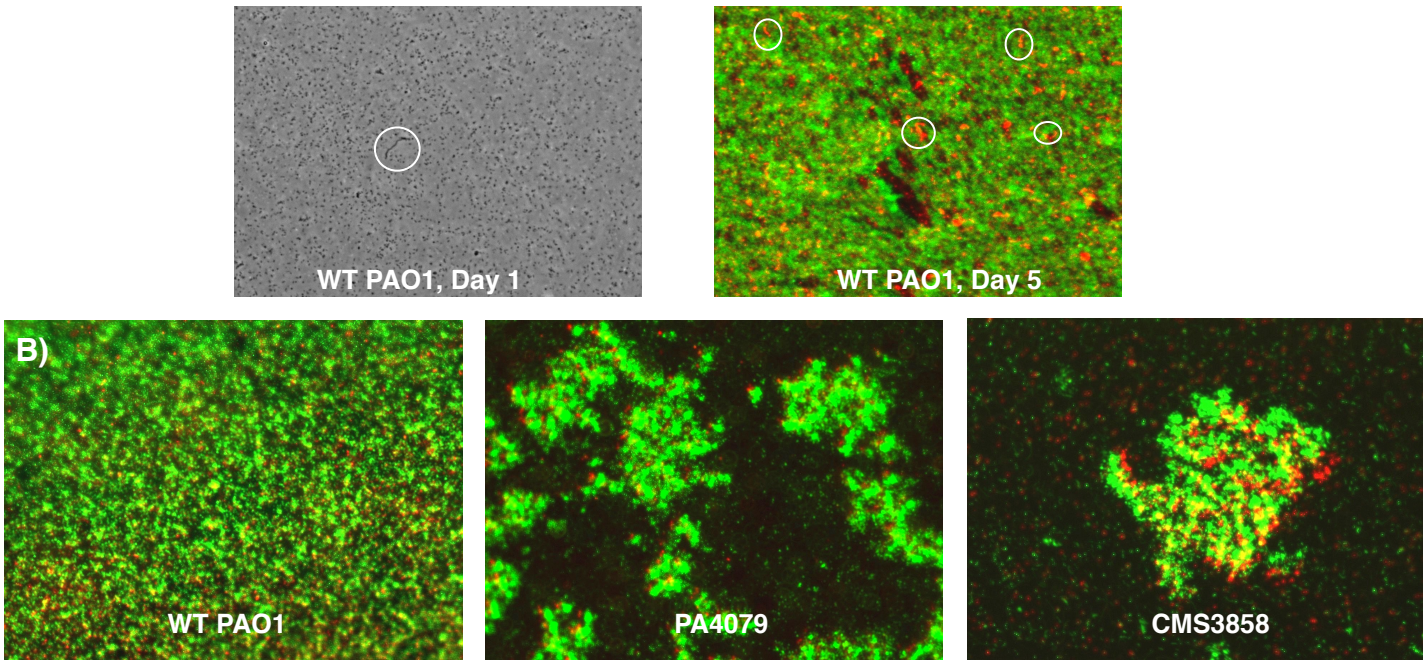


Figure 13: Static Biofilm Growth Results - Minimal Media, 37°C. **A)** Filamentous structure was observed only in wild-type biofilm throughout experiment, seen above on Day 1, then on Day 5 stained red. **B)** Wild-type PAO1 versus clumping phenotype seen in mutant biofilms.

3.2.2 Growth at 22°C - Varied Media

Results at 22°C for both LB broth and supplemented M9 minimal media were similar to those seen at 37°C. In LB broth, both mutants again formed thread-like, wispy biofilms whereas the wild-type formed a typical biofilm lawn (Figure 14A). When grown in minimal media, both mutant strains showed the clumping, aggregate phenotype as seen in at 37°C when compared to the wild-type biofilm (Figure 14B, Pg. 25). These results lead us to believe that the PA4079 gene influences phenotypic expression when grown in static conditions.

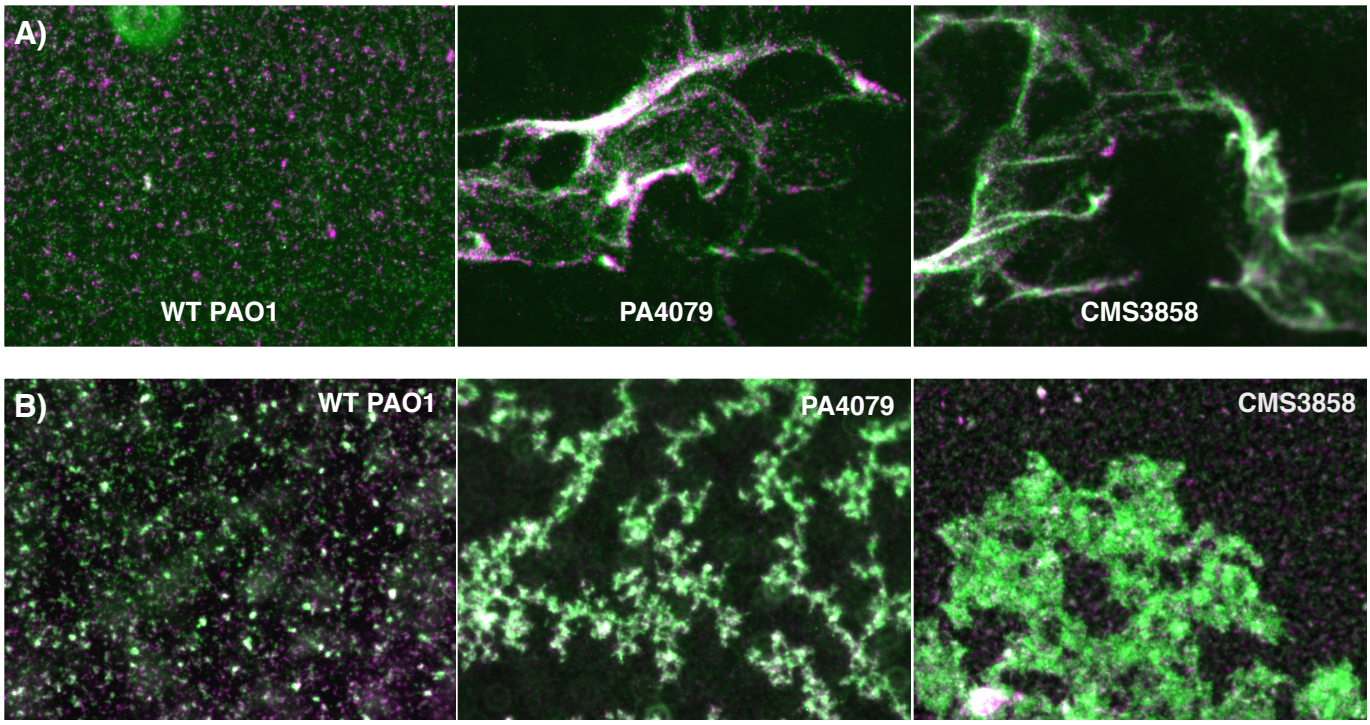


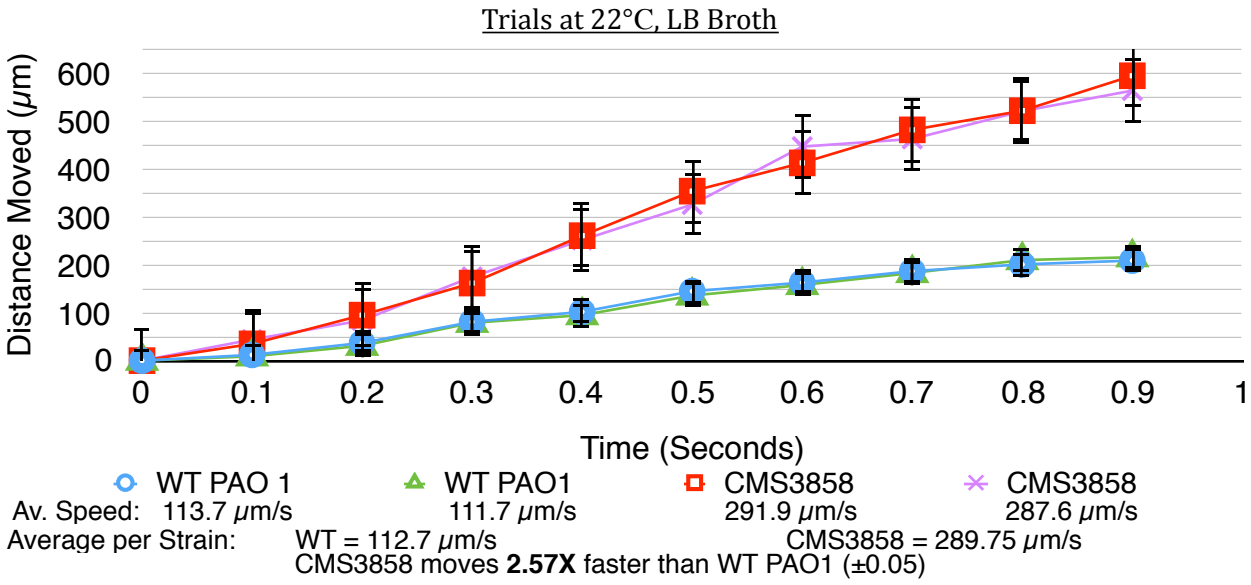
Figure 14: Static Biofilm Growth Results - 22°C. **A)** Day 4, LB Broth. Wild-type PAO1 consistent biofilm lawn compared to thread-like phenotype expressed within both mutant biofilms. **B)** Day 4, Minimal Media. Wild-type PAO1 versus clumping phenotype seen in mutant biofilms.

3.3 Dynamic Biofilm Growth - Flow Cell System

As discussed in Section 2, time-lapse photography was captured daily for each line during each experiment, and images were transformed to a stack in which the speed of individual cells could be quantified within the biofilms. Recall the strains used for flow cell system trials were wild-type PAO1 and the CMS3858 mutant (clean deletion of PA4079 gene). Using FIJI, individual cells were manually tracked and movement was plotted as distance moved over time with Excel. For quantification purposes, the conversions of 1 pixel = 1 µm were used to track the movement of cells within the daily time-lapse videos. Deviations were calculated by FIJI software.

For the trial conducted at 22°C with full-strength LB broth, visible differences in motility were seen beginning on Day 3 and were consistent to Day 5. It was visible through the time-lapse videos that CMS3858 cells moved faster than the wild-type cells in biofilms, which was calculated to be **2.57 and 5.08, and 1.82** times faster, as shown by the data on Days 3, 4, and 5 respectively (Figure 15). For the experiment at 22°C with 1/10X LB, videos showed that CMS3858 cells moved at roughly the same rate as the wild-type; speed was calculated to show that CMS3858 is moving **1.06 and 1.10** times faster that the wild-type on Days 3 and 5 respectively (Figure 16). Day 4 showed no difference in speed between the wild-type and CMS3858.

A)



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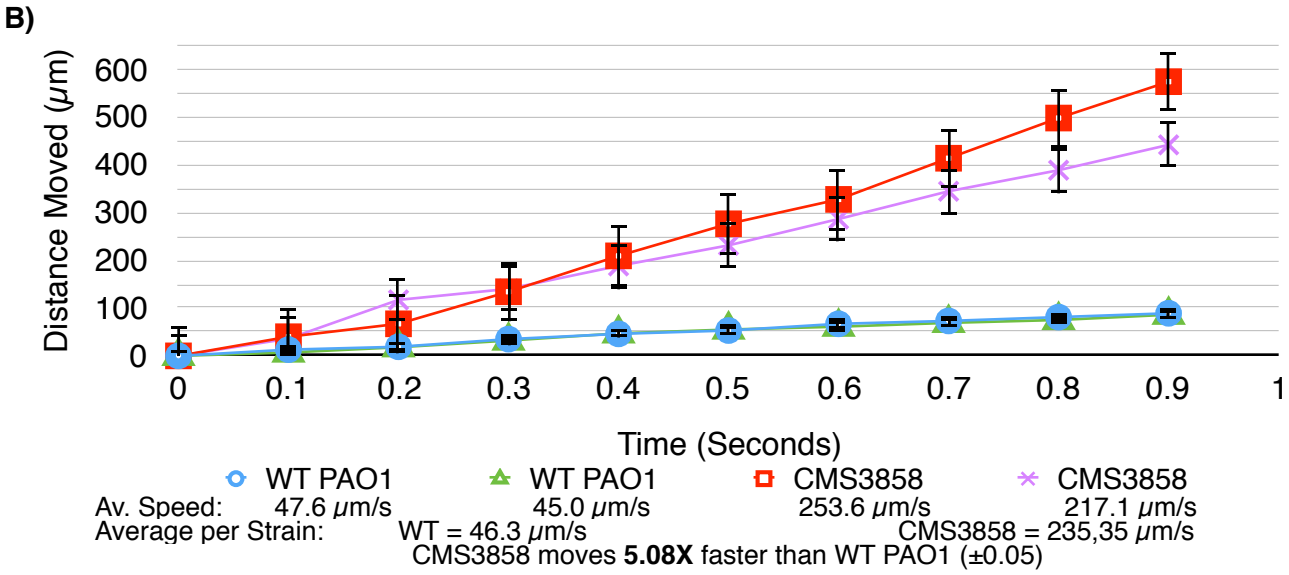

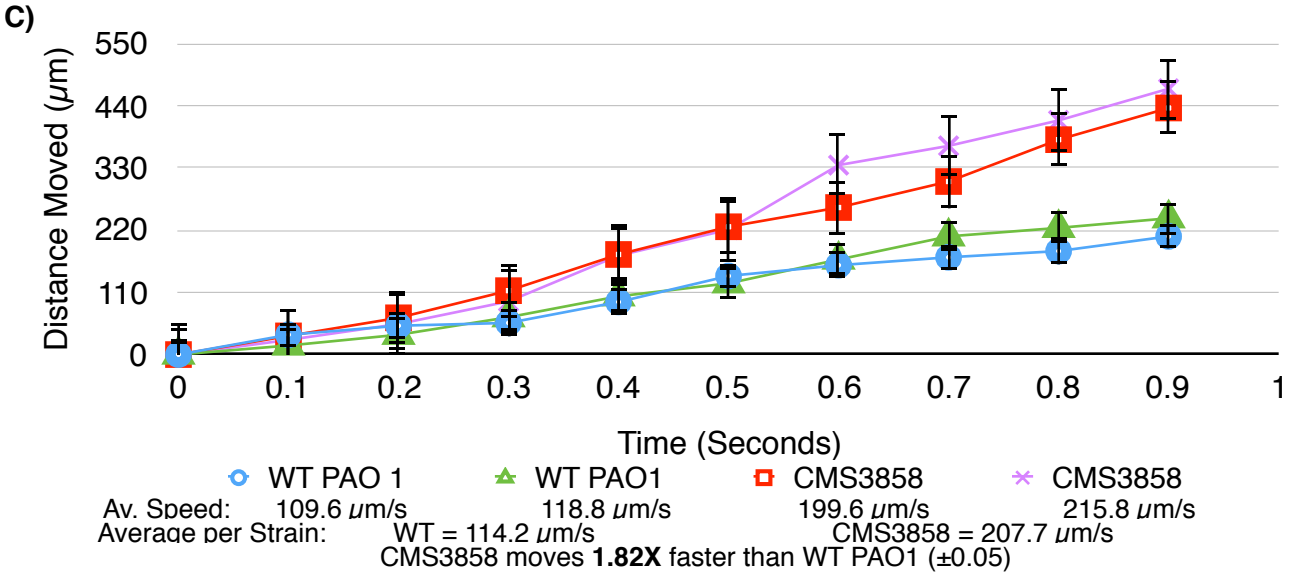

Figure 15: Speed Graphs for 22°C and LB. **A)** Day 3. **B)** Day 4. **C)** Day 5.

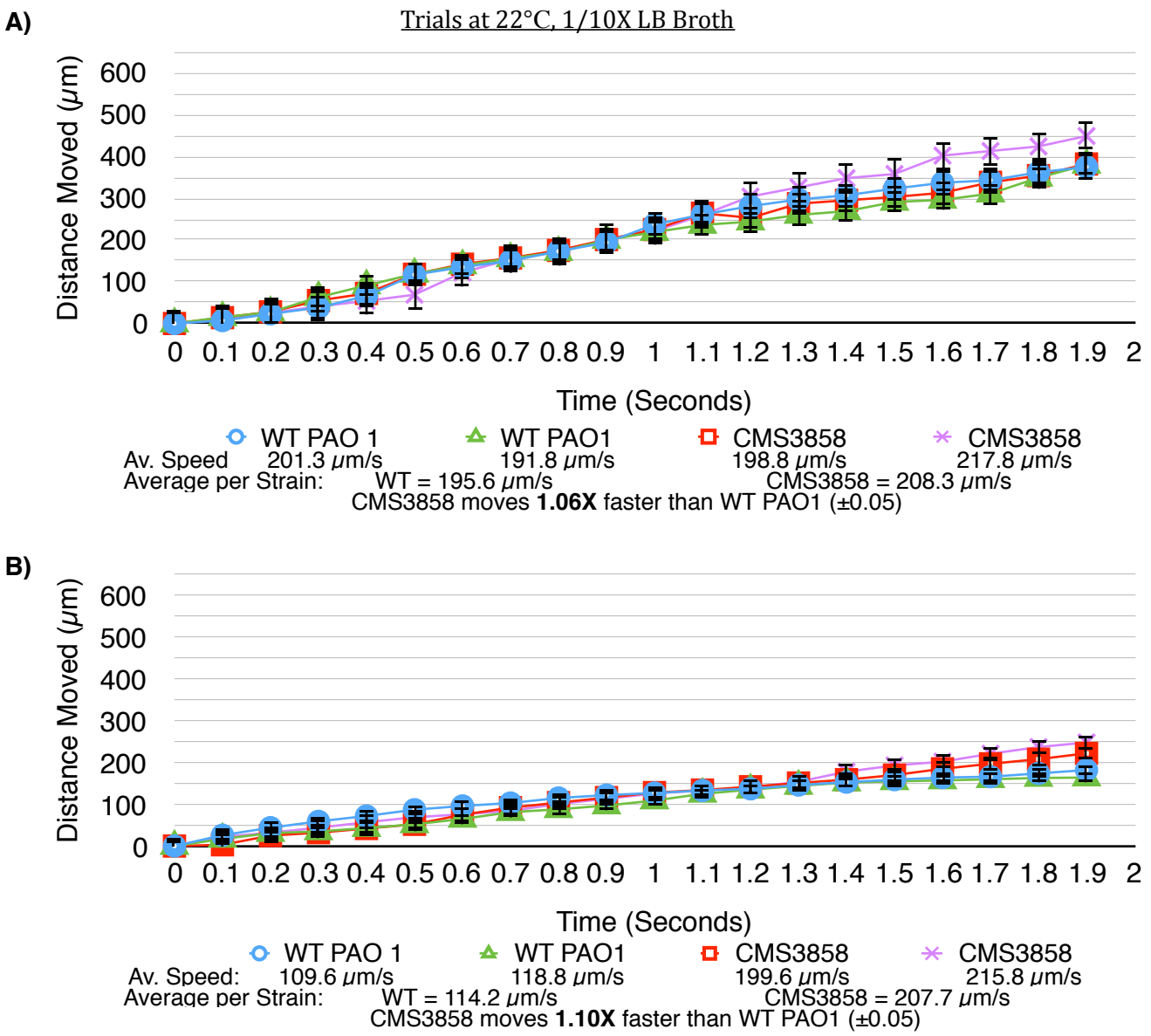

Figure 16: Speed Graphs for 22°C and 1/10X LB. **A)** Day 3. **B)** Day 5.

For the trial run at 37°C with full-strength LB broth, similar results were again similar to the experiment at 22°C with full-strength LB. Figure 17 shows the mutant CMS3858 cells move **1.64, 1.98, and 2.00** times faster than the wild-type. A trial at 37°C for the 1/10X LB broth was not conducted at this time due to a leak in the waste container and glass cells.

For accurate conclusions of the flow cell system experiments, this experiment will need to be completed and data will need to be included.

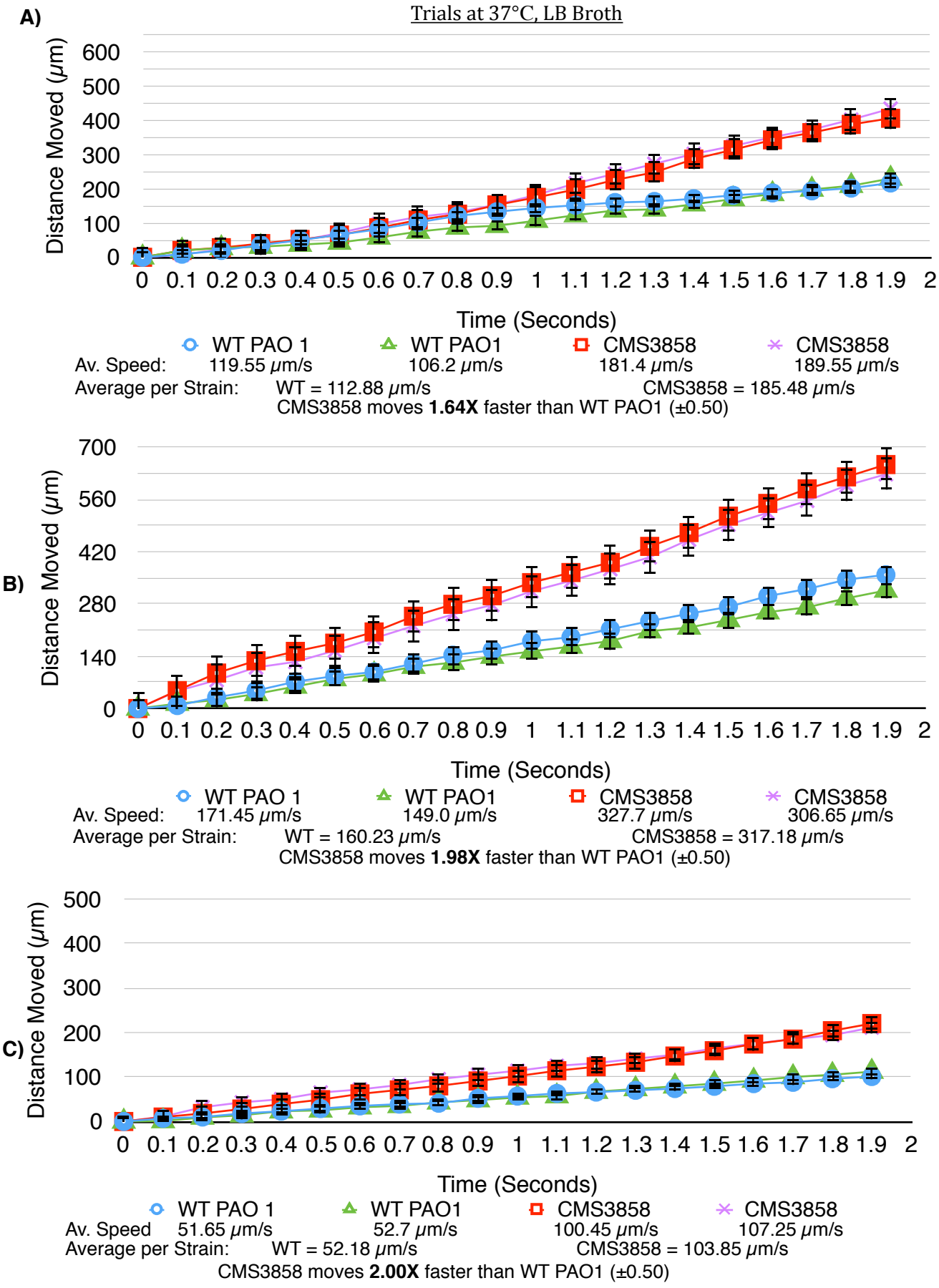

Figure 17: Speed Graphs for 37°C and LB. **A)** Day 3. **B)** Day 4. **C)** Day 5.

3.4 Pyocyanin Assays

No major differences were noticed in amount of pyocyanin produced between wild-type PAO1 and the two mutant strains. This was surprising, as both PA4079 and CMS3858 appeared to express more of the blue-green pigment when grown in liquid culture (Figure 10A). Experiments to quantify the amount of pyocyanin produced per strain yielded results too similar to prove a difference between the wild-type PAO1 and mutant strains exists. More experimentation and different assays need to be conducted to extract pyocyanin from these *P. aeruginosa* strains.

5. Conclusions and Future Works

Our group intends to further study the role of the PA4079 gene and other gene homologs in *P. aeruginosa* to determine which genes control social motility, and how these genes ultimately influence phenotypic expression within the formation of biofilms. Experiments will be conducted using strain CMS4293, which is the PA4079 gene complimented strain of CMS3858, to show that the PA4079 gene is contributing to the results seen in its absence. Trials using CMS4293 will determine if reintroducing the gene of interest restores wild-type function to the CMS3858 clean deletion mutant. An experiment with the flow cell system using 1/10X strength LB broth at 37°C should also be conducted to confirm inferences regarding growth rates and expression within a dynamic growth environment.

With the results seen throughout this investigation, it can be said that the PA4079 gene and protein homologs do have an influence on social motility and phenotypic expression within *Pseudomonas aeruginosa* biofilms. Although the presence of the PA4079 gene in wild-type *P. aeruginosa* appears to act as a motility repressor, the mode of inhibition has not been specified so further experimentation must be conducted. Understanding the social motility factors of *P. aeruginosa* as a model organism is promising for the treatment and prevention of infections caused by pathogenic biofilms, as seen in cystic fibrosis, wounds, and implants. Recognizing genes and homologs responsible for dispersal from pathogenic biofilms could present a possible target for new treatments.

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Appendix

UEGC Strains			Shanks Strains		
	0 Hour	24 Hour		0 Hour	24 Hour
(WT) PAO1	$1.9x10^6$	$4.2x10^9$	CMS4202	$1.9x10^6$	$3.7x10^9$
PA4079	$3.9x10^6$	$2.7x10^9$	CMS4292	$2.1x10^6$	$2.4x10^9$
CMS3858	$3.0x10^6$	$3.1x10^9$	CMS4293	$1.9x10^6$	$2.1x10^9$
(WT) PAO1 + pMRP9	$4.1x10^6$	$5.1x10^9$	CMS3960	$2.4x10^6$	$3.3x10^9$
PA4079 + pMRP9	$3.5x10^6$	$3.2x10^8$			
CMS3858 + pMRP9	$2.1x10^6$	$2.3x10^9$			

Table 1A: *P. aeruginosa* Isolates - Growth Curve CFU Counts. CFU counts show no significant differences in growth rates between all mutant strains at 37°C temperature in respective medias.

Day 3, 570 nm

	1	2	3	4	5	6	7	
1	1.9424	1.9402	1.8725	2.0937	1.7094	1.1728	1.2802	
2	2.1506	1.7917	1.4938	1.5895	1.0428	1.1347	0.9722	
3	2.4762	2.0407	1.5821	2.0848	1.4738	1.1726	1.2141	
Average	2.1897333	1.9242	1.6494666	1.9226666	1.4086666	1.1600333	1.1555	
SD	0.2690430	0.1252687	0.1981341	0.2885651	0.3380394	0.0219395	0.1621464	
							Avg.StDev	0.2004480

Day 3, 595 nm

	1	2	3	4	5	6	7	
1	1.4805	1.4724	1.426	1.5529	1.2945	0.9364	0.9607	
2	1.5605	1.3901	1.1616	1.2221	0.8646	0.9111	0.7808	
3	1.7866	1.5016	1.1848	1.5095	1.0991	0.9288	0.9256	
Average	1.6092	1.4547	1.2574666	1.4281666	1.0860666	0.9254333	0.8890333	
SD	0.1587547	0.0578189	0.1464143	0.1797734	0.2152461	0.0129816	0.0953616	
							Avg.StDev	0.1237644

Table 2A: Tables used to generate chart for Day 3 Crystal Violet Assay.

Day 5, 570 nm

	1	2	3	4	5	6	7	
1	2.9001	2.5827	2.3415	2.5208	2.5849	3.0519	2.5363	
2	2.4757	2.715	2.2706	2.9815	2.4242	1.8188	2.5502	
3	2.6508	2.1551	2.6524	2.7549	3.0621	1.8534	2.5598	
Average	2.6755333	2.4842666	2.4215	2.7524	2.6904	2.2413666	2.5487666	
StDev	0.2132783	0.2926411	0.2030832	0.2303601	0.3317782	0.7021556	0.0118153	
							Avg.StDev	0.2835874

Day 5, 595 nm

	1	2	3	4	5	6	7	
1	2.2016	1.9483	1.7971	1.9291	2.0157	2.3528	1.9753	
2	1.8801	2.0459	1.7142	2.2379	1.8635	1.3716	1.9214	
3	1.9866	1.6347	1.9999	2.135	2.333	1.4481	1.9539	
Average	2.0227666	1.8763	1.8370666	2.1006666	2.0707333	1.7241666	1.9502	
StDev	0.1637729	0.2148472	0.1469834	0.1572369	0.2395392	0.5457544	0.0271398	
							Avg.StDev	0.1869092

Table 3A: Tables used to generate chart for Day 5 Crystal Violet Assay.

		Day 3		
Time (Sec)	**WT PAO1**	**WT PAO1**	**CMS 3858**	**CMS 3858**
0	0	0	0	0
0.1	12	9	35	44
0.2	37	31	95	84
0.3	81	79	162	175
0.4	102	95	261	252
0.5	145	136	354	326
0.6	163	158	413	447
0.7	187	183	482	463
0.8	201	210	522	521
0.9	209	216	595	564
Avg	113.7	111.7	291.9	287.6
Avg / strain	112.7		289.75	
		289.75/ 112.2 = 2.57		

		Day 4		
Time (Sec)	**WT PAO1**	**WT PAO1**	**CMS 3858**	**CMS 3858**
0	0	0	0	0
0.1	13	7	40	36
0.2	19	18	67	117
0.3	35	32	134	140
0.4	46	47	209	188
0.5	53	55	276	231
0.6	67	61	327	286
0.7	73	69	413	344
0.8	81	75	497	388
0.9	89	86	573	441
Avg	47.6	45	253.6	217.1
Avg / strain	46.3		235.35	
		235.35/ 46.3 = 5.08		

		Day 5		
Time (Sec)	**WT PAO1**	**WT PAO1**	**CMS 3858**	**CMS 3858**
0	0	0	0	0
0.1	35	16	33	26
0.2	51	35	65	54
0.3	56	66	113	94
0.4	93	103	177	175
0.5	139	126	226	221
0.6	158	168	260	335
0.7	172	209	306	369
0.8	183	224	380	414
0.9	209	241	436	470
Avg	109.6	118.8	199.6	215.8
Avg / strain	114.2		207.7	
		207.7/ 114.2= 1.82		

Table 4A: Data for graphs of trials at 22°C in LB broth.

		Day 3		
Time (Sec)	**WT PAO1**	**WT PAO1**	**CMS 3858**	**CMS 3858**
0	0	0	0	0
0.1	7	15	14	12
0.2	23	27	28	25
0.3	38	63	55	41
0.4	66	91	72	54
0.5	117	119	118	69
0.6	135	139	142	122
0.7	150	156	157	154
0.8	173	171	175	172
0.9	194	200	201	202
1.0	237	219	225	221
1.1	261	236	264	260
1.2	281	244	253	304
1.3	297	260	287	326
1.4	307	269	295	348
1.5	323	291	303	358
1.6	337	296	313	402
1.7	343	311	338	413
1.8	362	349	354	424
1.9	375	380	382	449
Avg	201.3	191.8	198.8	217.8
Avg / strain	196.55		208.3	
		208.3/ 196.55 = 1.06		

		Day 5		
Time (Sec)	**WT PAO1**	**WT PAO1**	**CMS 3858**	**CMS 3858**
0	0	0	0	0
0.1	26	17	3	22
0.2	44	31	25	32
0.3	59	35	32	44
0.4	72	43	42	57
0.5	87	52	52	69
0.6	96	65	75	76
0.7	103	81	92	84
0.8	115	88	104	101
0.9	122	97	116	113
1.0	127	109	128	126
1.1	133	125	134	133
1.2	137	135	142	140
1.3	146	144	151	154
1.4	153	152	159	178
1.5	159	155	170	193
1.6	164	157	185	202
1.7	166	160	197	221
1.8	174	163	208	237
1.9	182	164	222	248
Avg	113.25	98.65	111.85	121.5
Avg / strain	105.95		116.675	
		116.675 / 105.95 = 1.10		

Table 5A: Data for graphs of trials at 22°C in 1/10X LB broth.

		Day 3		
Time (Sec)	WT PAO1	WT PAO1	CMS 3858	CMS 3858
0	0	0	0	0
0.1	9	20	20	7
0.2	20	27	28	24
0.3	36	31	40	38
0.4	50	37	52	49
0.5	66	43	65	71
0.6	82	58	86	97
0.7	102	75	109	118
0.8	121	87	126	131
0.9	133	92	153	154
1.0	144	107	175	182
1.1	152	123	198	216
1.2	160	137	226	244
1.3	163	140	248	273
1.4	171	155	287	302
1.5	181	170	314	325
1.6	188	185	343	351
1.7	194	198	364	373
1.8	202	209	388	402
1.9	217	230	406	434
Avg	119.55	106.2	181.4	189.55
Avg / strain	112.875		185.475	
		185.475 / 112.875 = 1.64		

		Day 4		
Time (Sec)	WT PAO1	WT PAO1	CMS 3858	CMS3 858
0	0	0	0	0
0.1	8	13	48	47
0.2	30	23	95	76
0.3	48	39	128	110
0.4	72	59	153	125
0.5	87	79	175	155
0.6	98	92	205	188
0.7	120	112	247	221
0.8	143	123	279	252
0.9	156	139	302	277
1.0	180	153	337	314
1.1	191	166	364	342
1.2	212	181	391	373
1.3	234	207	434	405
1.4	254	217	471	452
1.5	272	237	515	492
1.6	301	258	549	524
1.7	320	271	588	556
1.8	345	295	620	597
1.9	358	316	653	627
Avg	171.45	149	327.7	306.65
Avg / strain	160.225		317.175	
		317.175 / 160.225 = 1.98		

		Day 5		
Time (Sec)	WT PAO1	WT PAO1	CMS 3858	CMS 3858
0	0	0	0	0
0.1	7	1	10	11
0.2	10	9	18	32
0.3	18	14	28	43
0.4	23	23	39	50
0.5	29	25	49	65
0.6	35	32	62	72
0.7	39	35	71	82
0.8	41	43	80	95
0.9	52	48	91	105
1.0	57	54	102	114
1.1	63	57	114	125
1.2	66	67	123	131
1.3	70	73	133	141
1.4	74	79	147	150
1.5	79	85	159	164
1.6	85	92	174	175
1.7	88	100	185	185
1.8	96	105	204	194
1.9	101	112	220	211
Avg	51.65	52.7	100.45	107.25
Avg / strain	52.175		103.85	
		103.85 / 52.175 = 2.00		

Table 6A: Data for graphs of trials at 37°C in LB broth.